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Review Article

Engineered Recombinant Protein Production Systems Originated from *Escherichia coli*

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HIGHLIGHTS

- Selection of a suitable host is an important step in recombinant protein production.
- *Escherichia coli* cells are the most attractive host in therapeutic protein production.
- Engineered strains had developed to produce active protein with suitable conformation in industrial scale.
- Classical mutagenesis & genome engineering are two important strain engineering strategies.

ABSTRACT

Selection of a suitable host strain is an important step in recombinant production of heterologous proteins. As the prokaryotic systems and especially *Escherichia coli* (*E. coli*) cells are the most attractive host in therapeutic protein production, various derivatives of this organism have been developed to overcome the limitations exist in recombinant protein production, such as inefficient expression and folding of target proteins with eukaryotic origin. This review summarized key strategies for *E. coli* engineering and introduced some new and applicable engineered *E. coli* strains that produce more complex proteins for therapeutic and research use in the future.

Introduction

Recombinant proteins have vast applications in research, diagnosis and therapeutic fields (Andersen and Krummen, 2002; Biswal et al., 2016). In general, specific protein isolation from natural sources as had done in the past, is an inefficient and laborious work as well as having safety and availability challenges. Introduction of recombinant technology get rid of these limitations through cloning the protein coding DNA in an appropriate vector and foreign expression in a suitable host. Nowadays, different expression systems use for recombinant protein production , including mammalian cells like CHO, bacterial cells like *Escherichia coli*, yeast, cell-free system, transgenic plants and animals (Ranjbari et al., 2015). *Escherichia coli* is preferable choice for production of recombinant proteins both on a lab scale and in industry, as at this time, around one third of the approved therapeutic proteins are being produced in this prokaryotic host (Baeshen et al., 2015). Owing to its low cost, the ease of genetic manipulation, rapid growth, high yield of the product, cost effectiveness, and easy scale-up process, *E. coli* is considered as the first choice for industrial large-scale production of therapeutic proteins, particularly non-glycosylated ones (Jia and Jeon, 2016). Other attractive features for industrial applications are the availability of various *E. coli* expression vectors and strains, relatively easy protein folding mechanisms, and bioprocess technologies. The limitation in post translational modifications like glycosylation, acetylation, phosphorylation, and proteolytic processing, restrict its application in the production of slightly complex eukaryotic proteins (Jia and Jeon, 2016). Several technological strategies have emerged new features in the *E. coli* expression system and resulted in several high

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performance strains. These novel engineered strains, as the genetically modified *E. coli* can express complex proteins, including growth factors or full-length glycosylated antibodies (Löffler et al., 2016). Recent studies have demonstrated that these promising genetically engineered strains of *E. coli* enhanced markedly the production capacities for recombinant proteins.

Important considerations for recombinant protein production using *E. coli*

There are different strategies in *E. coli* engineering that result in improved protein synthesis and/or folding. Rather than strain selection, some other concerns have to be considered in an efficient protein production in *E. coli* using recombinant DNA technology. Some of these concerns are:

Codon Usage in E. coli

Genes that contain rare codons of *E. coli* may be inefficiently expressed by this organism, as the amount of each transfer RNAs (tRNAs) is dependent on the frequency of that codon in natural genomic content of organism. Therefore, codon optimization is an essential step of process design to increase the expression level of heterologous recombinant proteins. In this approach synonymous codon mutations are performed in messenger RNA (mRNA) coding regions. Most amino acids can be encoded by more than one synonymous codon. Appropriate codon usage in the design of coding gene can have a distinct influence on protein expression efficiency (Sharp et al., 1988; Burgess-Brown et al., 2008; Mauro, 2018). Ghavim *et al.* demonstrated that codon optimization at N-terminal of the recombinant human growth hormone gene could significantly increase the expression of human growth hormone in *E. coli* (Ghavim et al., 2017).

Enhanced mRNA stability

The half-life of mRNA can effect on translation and protein synthesis. So, in *E.coli* such other bacteria, mRNA stability is rate limiting step in translation and, hence, in recombinant protein production (Makino et al., 2011). RNase E is an essential *E. coli* endonuclease, which has an important role on mRNA degradation at C-terminal region. Mutation in this region decrease the activity of RNase E, and therefore, increase mRNA stability and consequent protein expression (Lopez et al., 1999).

Chaperone co-expression strategy

The biological activity of proteins is dependent on their three-dimensional fold. However, in the cellular environment, newly synthesized proteins are at great risk of misfolding and aggregation. To avoid these risks, cells provide a collection of molecular chaperones. A major role of these chaperones is to protect newly synthesized polypeptide chains and also, assembled subunits from aggregating into nonfunctional structures. Recombinant protein expression in *E. coli* often results in the formation of inclusion bodies which are the insoluble aggregates form of proteins. Co-expression of chaperone can be a good approach from several techniques applied for the prevention of inclusion bodies formation. Chaperones assist newly synthesized proteins fold into their native conformation and shield exposed hydrophobic patches involved in the initiation of protein aggregation (Hartl et al., 2011; Kim et al., 2013).

Expression of disulfide-bonded proteins using E. coli

The other challenge with the production of recombinant proteins in *E. coli* is formation of favored disulfide bonds. The cytoplasm of *E. coli* is usually retained in reduced state and the activity of thioredoxin and glutaredoxin/glutathione enzyme systems result in the prevention of disulfide bonds formation. Also, the protein of interest is expressed in the cytoplasm in the form of inclusion bodies, in most cases, and needs to be solubilized and re-folded *in vitro*. Some strains with mutations in thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes have oxidizing condition in cytoplasm and therefore, can enhance disulfide bond formation in the *E. coli* cytoplasm. It should be considered that these strains can not reduce ribonucleotides and therefore, need exogenous reductant, such as dithiothreitol (DTT) for growth (Stewart et al., 1998; De Marco, 2009; Gąciarz et al., 2017).

Engineered E.coli strains

The ultimate goal of microorganism engineering is to design strains that can produce active protein with suitable conformation in industrial scale. Strain engineering is a very promising approach in this regard that can evolve engineered *E. coli* strains with significantly improved features for efficient production of recombinant protein (Makino et al., 2011). Nowadays, scientists use different methods to produce strains with increased production capacity, decreased toxicity caused by overproduction, optimization of cellular pathways and physiology (Santos and Stephanopoulos, 2008). Classical mutagenesis and genome engineering methods are two important groups of strain engineering strategies. Some classical mutagenesis strategies in bacteria through the enhancement of recombinant protein production consist of spontaneous chromosomal mutagenesis (Miroux and Walker, 1996), chromosomal mutagenesis using chemical mutagens or mutator genes (Massey-Gendel et al., 2009), transposon

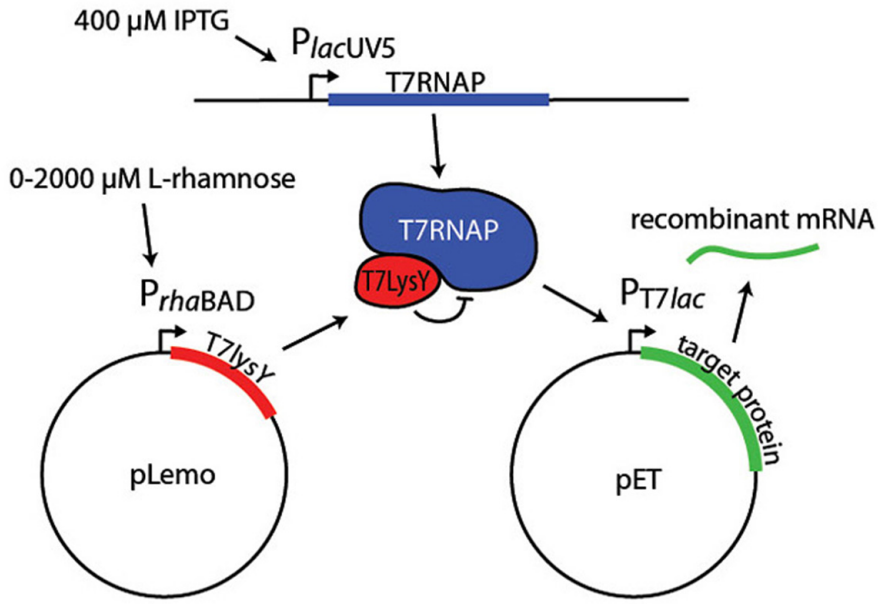

Figure1. Lemo21 (DE3) strain; different concentrations of L-rhamnose control T7RNAP and recombinant protein expression (Womack, 2011).

mutagenesis (Makino et al., 2011), and co-expression of the ASKA library (a complete set of *E. coli* K -12 ORF Archive) (Skretas and Georgiou, 2009). Also, there are some genome engineering strategies like, global transcription machinery engineering (gTME) (Alper and Stephanopoulos, 2007; Lee et al., 2008; Klein-Marcuschamer et al., 2009), Libraries of artificial zinc fingers (Park et al., 2003; Park et al., 2005; Lee et al., 2008), trackable multiplex recombineering (TRMR) (Warner et al., 2010) and genome shuffling (Patnaik et al., 2002; Zhang et al., 2002; Dai and Copley, 2004) which can enhance recombinant protein production in *E. coli*.

Lemo21 (DE3)

E. coli BL21 (DE3) is a common used strain to overexpress recombinant proteins. In this approach, the target protein gene is located under control of the T7 promoter of plasmid, and is recognized by the T7 RNA polymerase (RNAP). The expression of T7 RNAP gene on the chromosome is controlled by the non-titratable, IPTG-inducible lacUV5 promoter. In this system, proteins expression leads to inclusion bodies formation and their accumulation and in some case, uncontrolled protein expression can lead to the growth inhibition. So, as a general evidence, less expression of proteins generate more production of protein in the desired form, particularly for membrane protein expression (Wagner et al., 2008). Fine tuning of T7 expression can control the challenge of inclusion body formation and the toxic effect of recombinant proteins on growth. Lemo21 (DE3) is the next generation of *E. coli* that has more features than BL21(DE3), can serve tunable expression of challenging clones. Tunable expression is achieved by changing lysozyme (lysY) level, as the natural inhibitor of T7 RNA polymerase, by adding L-rhamnose to the expression culture in the range of 0 to 2000 µM (Fig. 1) (Schlegel et al., 2012). Schlegel *et al.* studied the effect of different concentrations of rhamnose on the expression of membrane proteins in Lemo21 (DE3) and observed that this strain could cause overexpression of these proteins and may be very suitable for optimizing the production of membrane proteins (Schlegel et al., 2012). Lemo21(DE3) can highly expressed membrane proteins at different concentrations of rhamnose. Lemo21 (DE3) is run similar to a pLysS containing strain when grow in the absence of rhamnose. However, optional addition of rhamnose is led to the favored expression of the specific protein. Tuning the expression level of less soluble proteins, may also result in more soluble, properly folded protein (Wegerer et al., 2008; Marschall et al., 2017).

NiCo21 (DE3) strain

This strain is derived from BL21 (DE3) strain applied in some specific recombinant production containing metal binding domain. Immobilized metal affinity chromatography (IMAC) is a method of choice for isolation of recombinant protein produced as a fusion protein containing a metal binding domain in *E. coli* strains. For

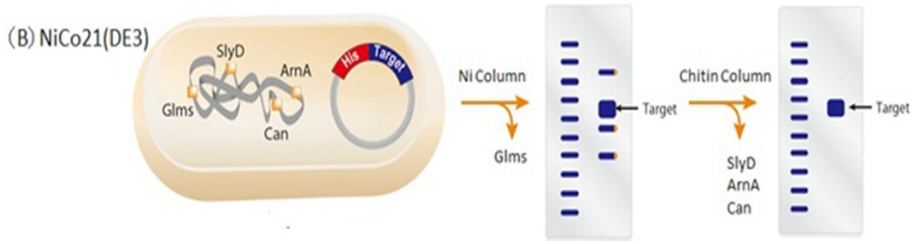

Figure 2. Two-step purification of histidine-tagged protein after expression in NiCo21 (DE3) (extracted from https://www.nebj.jp/products/detail/1087).

example, polyhistidine tag (6–10 consecutive His amino acids) offers high affinity to immobilized metals such as nickel and cobalt and routinely leads to a highly purified protein with greater than 80% purity, through a single metal affinity column. *E. coli* and other expression hosts have numerous essential proteins with metal cofactors. These endogenous metal-binding proteins interfere with the recombinant protein in the binding to IMAC resins and complicate isolation of the desired target protein and as a result, final product is contaminated with significant amounts of endogenous *E. coli* metal binding proteins (Robichon et al., 2011).

The NiCo21 (DE3) is an engineered protein expression strain with minimized *E. coli* protein contamination. In this strain, Glutamine--fructose-6-phosphate aminotransferase (GlmS) gene is mutated and three other proteins (SlyD, ArnA and Can) are tagged by chitin-binding domain (CBD) originating from *Bacillus circulans,* that eliminate cross contamination of IMAC resin with endogenous protein and enable rapid purification of SlyD, ArnA and Can protein by chitin affinity chromatography (Fig. 2) (Bolanos-Garcia and Davies, 2006; Robichon et al., 2011).

Robichon *et al.* designed and characterized two *E. coli* BL21(DE3) derivatives, NiCo21(DE3) and NiCo22(DE3), which express the *E. coli* endogenous proteins SlyD, Can, ArnA, and AceE (optionally) as fusion proteins with a chitin binding domain (CBD) and the protein GlmS, with six surface histidines replaced by alanines. *E. coli* CBD-tagged proteins were eliminated from the IMAC elution fraction using a chitin column, while the modification of GlmS results in loss of affinity for nickel-containing resin. These engineered strains are thus preferred expression strains for obtaining recombinant His-tagged target proteins with no host protein contamination and increased purification level (Robichon et al., 2011).

NEB Express Competent E. coli

NEB Company introduced a chemically competent *E. coli* strain suitable for high efficiency transformation and protein expression. Transformation efficiency of NEB strain is 0.6-1 x 10^9 cfu/µg of pUC19 DNA. This strain is deficient in proteases of Lon and OmpT and can be suitable for non-T7 protein expression. Different NEB stains offer variable levels of expression control, resistant to phage T1 and also, show extremely high transformation efficiencies (Samuelson, 2011). *E. coli* ER2523 (NEB Express) is an enhanced BL21 derivative available with or without the added control from *lacIq*. This engineered strain is recommended host strain for pMAL™ Protein Fusion and Purification System. The pMAL™ Protein Fusion and Purification System clone the desired gene in a pMAL™ vector, down-stream from the *malE* gene, which encodes maltose-binding protein (MBP). Therefore, an MBP-target fusion protein is expressed and then, purified by one-step affinity purification, which show specific binding to MBP. This technique uses the strong *Ptac* promoter and the translation initiation signals of MBP results in large amount expression of the fusion protein (Pryor and Leiting, 1997; Kapust and Waugh, 1999).

Rossetta strain

One of BL21 derivatives that have designed to enhance the expression of eukaryotic proteins is Rossetta strain containing codons rarely used in *E. coli*. Rare tRNAs for AGG, AGA, AUA, CUA, CCC and GGA codons by their native promoters are supplied with a compatible chloramphenicol-resistant plasmid. Thus, the Rosetta strains overcome the challenge of codon optimization for ″universal″ protein translation in *E. coli*. In Rosetta (DE3) pLysS, the rare tRNA genes are supplied with the same plasmids composed the T7 lysozyme gene. The strain of DE3 (a lysogen of λDE3) carries a chromosomal copy of the T7 RNA polymerase gene under control of the lacUV5 promoter and therefore, can be suitable for protein production using pET vectors and induction with IPTG (Tegel et al., 2010; Gopal and Kumar, 2013).

Origami strain

Origami host strains as *E. coli* K-12 derivatives have mutations in the thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes. This engineered strain exhibited enhanced disulfide bond formation in the *E. coli* cytoplasm. The Origami strains are kanamycin sensitive. These strains are recommended only for the production of proteins with essential disulfide bonds as a perquisite feature of proper folding, which avoid from undesired disulfide bond formation between polypeptides. There are different type of Origami strain base on different mutation and specific genes, like DE3, B(DE3), pLysS. The Origami B strains have combination of the desirable characteristics of BL21 and Origami strains. B host strains have mutations in trxB and gor as the original Origami strains. They also have a lacZY mutation compared to BL21 that enable precise control of expression levels by adjusting the concentration of IPTG. DE3 strains are a lysogen of λDE3 and have a chromosomal copy of the T7 RNA polymerase gene under control of the lacUV5 promoter. pLysS strains express T7 lysozyme, which suppresses basal expression of T7 RNA polymerase prior to induction and affect cell growth and viability (Xiong et al., 2005; Berkmen, 2012; Gopal and Kumar, 2013; Ranjbari et al., 2015).

BL21-CodonPlus

BL21-CodonPlus strains are engineered to contain extra copies of genes that encode the rare tRNAs, which limit translation of heterologous proteins in *E.coli*. The BL21-CodonPlus (DE3) strains utilize the T7 RNA polymerase promoter to enhance expression level. There are different codon plus strains, depend on the type of tRNAs encoded. BL21-CodonPlus-RIL contains extra copies of the argU, ileY, and leuW tRNA genes and encode tRNAs that recognize the arginine codons AGA and AGG, the isoleucine codon AUA, and the leucine codon CUA. These codons are useful for the expression of proteins originated from organisms with AT-rich genomes. BL21-CodonPlus-RP and BL21-CodonPlus (DE3)-RP cells contain extra copies of the argU and proL genes, encode tRNAs that recognize the Arg codons AGA and AGG and the Pro codon CCC, suitable for the production of proteins from organisms that have GC-rich genomes. Both strains of BL21-CodonPlus-RIL and BL21-CodonPlus-RP can be used with vectors containing non-T7 promoters. BL21-CodonPlus (DE3)-RIPL cells contain extra copies of the argU, ileY, and leuW as well as the proL tRNA genes, that can be utilize for heterologous proteins from organisms with AT- or GC-rich genomes (Kleber-Janke and Becker, 2000; Wu et al., 2004; Yin et al., 2007).

Conclusion

According to the different novel improved prokaryotic expression systems that are currently used in recombinant production of proteins, selection of an appropriate strain regard to the aim of study and protein identity, has an important effect on expression efficacy in yield and final bioactivity of expressed proteins.

Competing Interests

The author declared that there is no conflict of interest.

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